HIGH PRESSURE PROCESSING OF ORANGE AND GRAPEFRUIT JUICES

By

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

HIGH PRESSURE PROCESSING OF ORANGE AND GRAPEFRUIT JUICES

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The enzyme pectinesterase (PE) reduces the quality of citrus juices. Current commercial inactivation of the enzyme is accomplished by heat pasteurization. Pressurized treatments of orange and grapefruit juices to by-pass the use of extreme heat during processing is explored. PE inactivation using high pressure processing (HPP) in the range of 500-900 MPa was accomplished in orange and grapefruit juices. The higher pressures (>600 MPa) caused instantaneous inactivation of the heat labile form of the enzyme, but did not inactivate the heat stable form of PE. Heat labile grapefruit PE was also more sensitive than orange to pressure.

Isoelectric focusing and polyacrylamide gel

electrophoresis were used to isolate and examine extracted PE from Valencia juice. Untreated juice displayed a narrow, well defined band at 36,000 molecular weight. Juice treated at 90° C showed a complete loss of PE and no activity was detectable. Samples treated with HPP at 800 MPa or pasteurized at 70° C exhibited a decrease in PE activity and an aberration of the band at 36,000 molecular weight.

HPP was also investigated as a means to preserve cloud in fresh squeezed orange juice. Pressures from 500 MPa to 900 MPa were investigated at hold times of 1 second, 1 minute and 10 minutes. Cloud preservation was directly proportional to increased pressure and longer processing times. All treatments yielded a microbially stable product. A 90 day shelf life under refrigeration conditions was achieved using pressures of 700 MPa and higher combined with treatment times of at least 1 minute.

A storage study ascertained the difference in volatile profiles over time in high pressure treated juice versus traditional pasteurization. It was found that both statistical techniques were able to satisfactorily distinguish the two treatments from each other and the untreated control. Myrcene, α-pinene, sabinene, and d-limonene were the flavor compounds most affected by treatment.

INTRODUCTION

In order for food to have a longer shelf life, it must be processed to dramatically increase the stability over fresh, or unprocessed food. One of the most common and important techniques for prolonging shelf life and safety of food products is achieved by killing the microorganisms that cause spoilage and food borne illness by heat preservation. Often, enzymes that cause degradation after harvesting are also inactivated by thermal pasteurization or retort operations. However, heat processing can dramatically changes the fresh flavor and quality of the food product.

As an alternative to heat pasteurization, high pressure processing (HPP) has been shown to reduce microbial levels (Takahashi et al., 1993; Ogawa et al., 1992), affect properties and functionalities of proteins (Messens et al., 1997; Masson, 1992), and influence enzyme activity (Seyderhelm et al.1996; Basak and Ramaswamy, 1996). As such, it is rapidly gaining interest as a tool for food processing and an alternative to heat. Growth of pressure resistant bacterial spores is inhibited in high acid food, and thus, orange and grapefruit juices, which are commercially treated using heat, are prime candidates for HPP.

Current processing of citrus juice employs a pasteurization step, which has the purpose of reducing

microbial levels as well as inactivating pectinesterase (PE), the enzyme responsible for cloud loss in citrus juices during holding and storage. The severe levels of heating used in commercial pasteurization treatments are necessary to inactivate PE, and these are in excess of what is necessary to make the product microbially safe. Pasteurization results in a more stable product, but one that has also lost its more delicate volatile components. Heat treatment of orange juice can also lead to cooked off-flavors and caramelization of sugars, neither of which contributes positively to "good flavor." The first purpose of this project was to determine the effectiveness of high pressure for PE inactivation while maintaining flavor integrity. Since it has been generally observed that constituents of food can have a protective effect on the enzyme against inactivation by heat or pressure (Seyderhelm et al., 1996; Ogawa et al., 1990), PE was investigated in two of its biochemical environments: orange and grapefruit juice.

The second goal of this research was to determine the cloud stability of pressure treated juice versus a non-pressurized control. Juice cloud loss is the result of demethylated pectin interacting with calcium ions, causing a precipitation into a clear serum layer on top of a viscous layer of settled pectin and insoluble solids. Cloud is

retained by protecting the natural pectin in the extracted juice from enzymatic deesterification and degradation by pectinesterase (Rouse and Atkins, 1952). Turbidity is a desired characteristic of citrus juice, and cloud content is one of the criteria of quality (Rothschild and Karsenty, 1974). Thus, cloud loss is considered a quality defect in citrus juice, and inactivation of pectinesterase is one of the main reasons for the level of heating in commercial pasteurization. In this work, high pressure treatment is shown to inactivate the heat labile form of pectinesterase in orange and grapefruit juices. Since some PE activity remains in juice after pressurization, it was of interest to determine the stability of the cloud after pressure treatment.

The final goal of this project was to ascertain if high pressure treatment of fresh orange juice was discernable from fresh and pasteurized juice. Volatiles were monitored by purge-and-trap gas chromatography (GC) during storage to determine degradation of volatiles over time.

BACKGROUND

Physical Effects

The idea of controlling microorganisms by high pressure treatment is not novel. At the turn of the century, scientists had discovered that pressure extended the shelf life of milk by reducing the initial bacterial load by 5 to 6 log cycles (Hite, 1899). This author also investigated fruit juices and vegetables (Hite et al.,1914). It is now proposed that HPP affects the secondary bonds of large molecules such as proteins, sugars, and cell membranes (Mertens, 1996). Only recently has high pressure been considered as a food processing technique. Two companies already have pressurized products (e.g. jam, citrus juices, and yogurt) on the market in Japan.

Much of the work in this area to date has dealt exclusively with the control and inactivation of microbial populations through pressure treatment (Ogawa, et al., 1990, Shigehisa et al., 1991, Styles et al., 1991, Takahashi et al., 1991; Butz et al., 1990; Carlez et al., 1993, Ludwig et al., 1992; Raffalli et al., 1994; Sato et al., 1994; Smelt and Rijke, 1992; Yasumoto et al., 1993), so there is less known about chemical changes and enzyme inactivation due to

high pressure processing. One study has evaluated the changes in mandarin orange juice associated with high pressure treatment, both microbial and chemical(Takahashi et al., 1993). They found what they termed an insignificant change in a list of volatiles in Satsuma mandarin single strength juice.

A thorough analysis of physical characteristics after pressure treatment was reported, where standard methods were used to determine microbiological activity, chemical composition, pH, color, aroma and viscosity (Donsi et al., 1996). These results show no substantial compositional change in vitamins, sugars, pH, organic acids and several aroma compounds. Microbial inactivation was achieved at 350 MPa. Only viscosity was measurably affected by pressure treatment; a reduction in non-Newtonian viscosity (the ratio of shear stress and the corresponding velocity gradient) was noted.

Operating Principles

Two general scientific principles govern the action of high pressure on food. The first is LeChatelier's principle, which states that a reaction equilibrium will shift to minimize the effect of an external force applied to

the system, such as heat or the addition of product or reactant. This means a shift in the reaction resulting in a smaller volume will be enhanced by high pressure treatment, including chemical reactions as well as possible changes in molecular conformation. The second theory important to understanding the effect of high pressure is Pascal's principle, stating that as pressure is applied it travels instantaneously and uniformly throughout the sample. Pascal's principle is true regardless of the sample size or volume, which means that the whole sample (or food) will be treated uniformly throughout. This is in direct contrast to thermal processing, which results in hot spots and overheating of the surface to obtain the desired temperature in the center of the sample. The energy required to process by pressure is also lower since no additional energy is required to maintain the desired temperature level once it has been reached. This makes process time irrelevant to energy concerns, unlike heating. For reference, approximately the same energy is necessary to reach 400 MPa and 30 °C from ambient conditions(Cheftel, 1995).

Citrus juices are mostly comprised of water and thus will experience compression of about 15% at 600 MPa, increasing density and viscosity and therefore decreasing coefficients of diffusion. This directly affects reaction rates, as oftentimes with enzymes the diffusion of the two

reactants is the rate limiting step. According to the ideal gas law,

$$PV=nRT \qquad \text{EQ. 1}$$

where P is pressure, V is volume, n represents the number of moles, R is the ideal gas constant and T is temperature pressurization without a corresponding decrease in volume will result in an increase in the temperature of the sample. This temperature increase is not adiabatic, since there can be heat exchanged with the vessel wall. When pressure is released the sample loses the heat it gained through pressurization. A sample will end up close to its starting temperature after pressurization. Morild (1992) described the temperature change due to pressure changes as 1.86×10^{-3} K bar^{-1}. After adjusting the equation to the heat capacity of our pressure medium and converting to MPa, the conversion factor becomes 4.8×10^{-2} K MPa^{-1}. At the highest pressure used in this study, 900 MPa, the maximum theoretical temperature increase is 43.2 K (43.2 °C). This absence of temperature abuse in treated samples is the strongest case for using high pressure to pasteurize food.

Contributing Factors

Weak acids and bases dissociate under high pressure and pH changes can be expected in unbuffered samples (Lüdemann, 1992). Changes in pH can greatly affect the microorganisms and enzymes present in a food system and this must be considered when attributing any type of inactivation to high pressure alone. Greater inactivation of pectinesterase occurs at lower pH by pressure treatment (Ogawa et al., 1990) and heat treatment (Rouse and Atkins, 1952).

Process temperature is a major contributor to the efficacy of high pressure in inactivating enzymes and microorganisms. Temperatures above 50 °C or between -30 and +5 °C during processing enhance inactivation (Cheftel, 1995). High sugar or salt content as well as low water activity all have protective effects on pressure targets and it has been observed that the inactivation effect of pressure on microorganisms and pectinesterase is decreased by increasing juice concentration (Ogawa et al., 1990), illustrating the importance of understanding how pressure works in real food systems.

Finally, the method of pressurization should be considered. Some researchers claim that repeated pressure cycling has a more devastating effect on microorganisms and enzymes than the same total time in just one cycle. Honma and Haga, (1991) observed a greater reduction in

microorganism contaminants in egg white using cycling. Curl and Jansen, (1950 a&b) showed higher inactivation of trypsin, chymotrypsin and pepsin using a multi-cycle process. However, pectinesterase inactivation has not been shown to benefit from repeated pressure cycles (Irwe and Olsen, 1994). A cycle is defined by having only one pressurization and depressurization step. Commercially, a large benefit would be necessary to offset the increased wear on the pump and the pressure vessel, since increasing or decreasing pressure rather than holding at high pressure that wears out the pump and fatigues the vessel.

Enzyme Inactivation

Although the mechanism of enzyme inactivation is still unclear, the most accepted theory at present is that covalent bonds are not disrupted by high pressure, while electrostatic and hydrophobic interactions are. Thus, the primary structure of the enzyme remains unchanged, but the secondary and tertiary structure, and hence the active site on the enzyme, is susceptible to disorder. Even small changes in the active site can result in loss of enzyme activity. Four kinds of enzyme inactivation have been detailed (Miyagawa et al.1964): 1) completely and irreversibly inactivated, 2) completely and reversibly

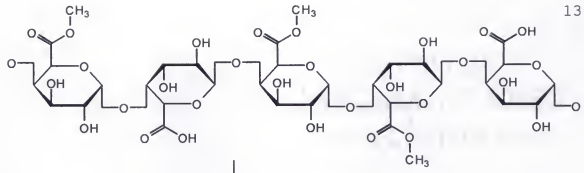
inactivated, 3) incompletely and irreversibly inactivated and 4) incompletely and reversibly inactivated. The decrease in volume caused by enzyme denaturation is the result of both the rearrangement of conformational volume and of solvent molecules. If the disorder caused by high pressure is favorable enough, the enzyme protein will be permanently denatured and disabled, while in other cases the damage is reversible. For instance, no evidence of recovery of pectinesterase after pressurization of 400 MPa for 10 minutes was found(Ogawa et al., 1992). Enzymes stabilized by high pressure can be expected to have increased activity due to pressurization. As shown by Asaka and Hayashi, (1991) enzymatic browning due to polyphenoloxidase seems to be enhanced. Every enzyme will therefore have a characteristic response to high pressure treatment, and even the same enzyme derived from different sources can react to differing degrees. Peroxidase was shown to have non-similar inactivation profiles in orange juice and strawberry puree (Cano et al., 1997). Grape, strawberry, apricot and apple polyphenoloxidases are more pressure stable than the polyphenoloxidase found in mushroom and potato (Hendrickx et al., 1998). Pectinesterase, lipase, polyphenoloxidase, lipoxygenase, peroxidase, lactoperoxidase, phosphatase and catalase were investigated and showed a different inactivation coefficient for each enzyme treated under the

same pressure conditions and in the same buffer solution (Seyderhelm et al., 1997).

Pectinesterase

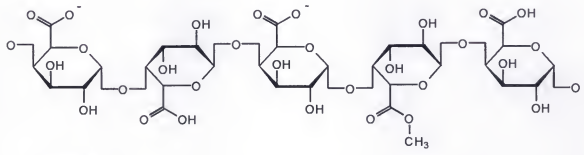
Pectinesterase (PE) is the enzyme in citrus juice responsible for cloud loss, a major quality defect in the final product. PE is found in all plant tissues and is tightly associated with the cell wall membrane. Intact citrus or other fruit is not rapidly degraded or softened by PE because of this. However, upon processing the cell wall matrix is disrupted, freeing the enzyme and allowing it to come in free contact with its substrate, pectin. Cloud loss occurs when the soluble pectin is deesterified and precipitated through complex formation with calcium ions. Figure 1 illustrates the pertinent reactions of pectin and pectinesterase which lead to cloud loss. At present, PE is thought to have two major forms, termed heat labile and heat stable. These two forms, or isozymes, of pectinesterase have different tolerances to heat treatment before inactivation occurs. Versteeg et al. (1980) defined the levels which could inactivate these two different forms as 70 °C and 90 °C. Seymour et al. (1991) purified and characterized two forms of PE and reported a higher molecular weight and higher percent hydrophobic amino acid content for the heat

Figure 1. Reactions of Pectin and Pectinesterase



Pectin

Pectinesterase
H_2O

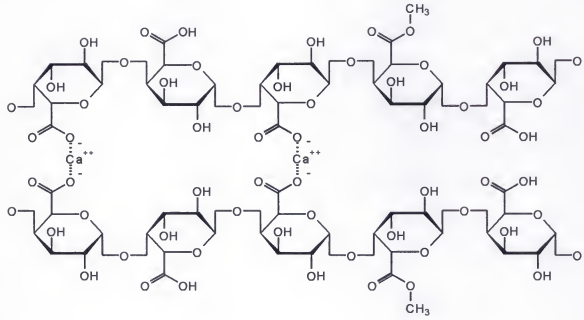


Pectic Acid

+

CH_3OH

Methanol



stable isozyme of pectinesterase. The heat stable portion is reported to represent about 10% or less of total PE activity in oranges (Versteeg et al., 1978).

Quality Effects

As mentioned before, pressurization of food involves some degree of heating of the sample. However, the temperature increase due to pressurization is minimal compared to traditional temperatures necessary to accomplish microbial and enzyme inactivation by heat alone. Thus, the quality of food products is more likely retained to a greater extent. High temperature, traditional thermal processing deteriorates color, flavor, nutrients and texture of foods. It is well documented that orange juice subjected to thermal pasteurization has a marked decrease in a perception of "fresh" flavor after thermal pasteurization. (Moshonas and Shaw, 1997; Nisperos-Carriedo and Shaw, 1990) For example, Yen and Lin (1996) found that pressurized guava puree retained its color, pectin, cloud and ascorbic acid content while successfully reducing microbial count to less than 10 colony forming units/mL. The microbial counts in the pressurized juice remained at this level during the storage trial of 60 days. Thermal pasteurization of the same puree sample showed a marked deterioration in the

measured quality aspects but was more successful at inactivating the enzymes present. Pasteurized samples were not as microbially stable as the samples pressurized at 600 MPa.

Kimura et al., 1994 determined that strawberry jam prepared by high pressure processing retained a higher quality regarding volatile flavors and natural color of the fresh fruit when compared to heat processed jam. Flavor components important to good strawberry flavor were determined to be in greater abundance in the pressurized sample immediately after treatment including: trans-2-hexenol (80 times) linalool (5 times), ethyl butyrate (6 times) 2-methylbutyric acid (7 times). In addition, a new "sweet" aroma was created in the heated jam. Browning occurs in heated jam during processing, but pressure treatment does not cause browning, and vitamin C was not lost due to pressure treatment as it was in the heated jam. The storage study accompanying this work showed the pressurized jam deteriorated more rapidly at room temperature. This was due to a higher dissolved oxygen content and the presence of active enzymes in the pressurized jam. Oxygen concentration decreased during the storage study, indicating that it was participating in various chemical and enzymatic reactions which contribute to deterioration. Also, the packaging material amenable to the two different types of processing

can cause some differences in storage stability regarding the oxygen content. Glass was used for the heated jam, and is a good gas barrier. Pressurization must take place in a flexible container and thus composite films are employed. Although these composite films were designated as gas barriers, they are still more permeable than glass to oxygen transfer. Pressures used in this study were 400-500 MPa, which is capable of inactivating microorganisms, but not particularly efficient at incapacitating enzymes. Higher pressures are necessary to achieve inactivation of enzymes consistent with a more shelf stable product.

Texture can be greatly affected by high pressure, especially in protein containing systems. Juices and food fragments do not show any documented textural changes, but whole fruits or large fruit pieces seem to suffer softening after pressure treatment. This is most likely due to cellular disruption and compression of internal gas vacuoles which lead to release of cell wall bound enzymes (Cheftel, 1992; Asaka and Hayashi, 1991). Protein solutions have been shown to gel when pressurized, and gels formed under pressure tend to have more tensile strength and a higher melting temperature (Gekko and Fukamizu, 1991). Conversely, muscle foods undergo tenderization during pressure treatment. Again, the cause has been attributed to release of intracellular enzymes with proteolytic activity (Ohmori

et al., 1991). It is left to the user to decide if the potentially texture altering effects of high pressure treatment are detrimental or beneficial in individual cases. Clearly, not all foods are ideally suited to this technology.

Separation Procedures

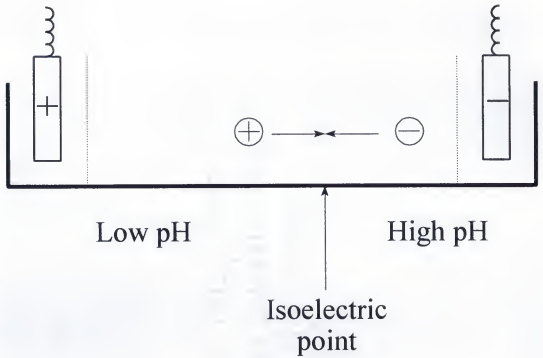
Isoelectric focusing (IEF) is a separation technique that uses a pH gradient superimposed along an electric field to separate components of a mixture based on their isoelectric points. An isoelectric point, or pI, is the pH at which the net charge on a molecule is equal to zero. A charged molecule, such as a protein, migrates toward the pole of opposite charge along the electrical gradient until it comes to the point in the pH gradient where its net charge will be zero. It comes to rest here, at its isoelectric point, which is nearly unique to every protein. If the molecule drifts from this zone, due possibly to diffusion, it becomes charged again and is simply pulled back to its pI by the electric field.

A pH gradient is established by a mixture of ampholytes with a pH range surrounding the pI of the compounds of interest. Each ampholyte has a different pI and migrates in

an applied electric field until it reaches the point where it is uncharged. Ampholytes can be mixed with the sample to be separated before applying the electric field. After migration has stopped, usually three to five hours, the mixture of proteins is separated with phenomenal sensitivity, sometimes within 0.003 pH units using immobilized gradients (Righetti et al., 1989). IEF is not just a separation technique, it also has the purpose of focusing the proteins into very narrow zones. This is an especially powerful technique when combined with gel electrophoresis to form a two dimensional separation.

Figure 2 represents the basic concept behind isoelectric focusing. The positively charged molecule migrates toward the cathode, while the negatively charged molecule is attracted to the anode. The pH gradient is set up with low pH at the anode and high pH at the cathode. As a molecule, for example, a positively charged protein, migrates toward the negatively charged pole, it encounters an increasingly more basic environment which mitigates the positive charge until it is zero. The protein will then stop migrating at this, its isoelectric point. Resolution of focused proteins is defined as the difference in pI or pH between clearly distinguishable bands, or fractions. Change in pI is proportional to the square root of the pH gradient, and inversely proportional to the square

Figure 2. Schematic Diagram of Iosoelectric Focusing System (Adapted from Giddings, 1991)


Low pH
High pH
Isoelectric
point

root of the field strength. To obtain optimal resolution, one would use the smallest range of ampholyte pH and the highest field strength. This must be balanced by the pI range of proteins being separated and the heating that results from high field strength. Heating will ultimately decrease resolution due to diffusion so field strength and run time must be optimized to minimize the deleterious effects of heating while providing a good separation.

MATERIALS AND METHODS

Pectinesterase Inactivation

Juice Preparation

Samples of orange and grapefruit juice were extracted in the pilot plant of the Citrus Research and Education Center in Lake Alfred, Florida using an FMC commercial extractor (FMC, Lakeland, FL). The juice was not subjected to a finishing step. Juice not immediately used for PE inactivation studies was stored frozen at -23 °C and thawed before use. Additional fresh frozen finisher pulp from previous juice runs was added after thawing on a weight basis at 10.7% for orange and 8.7% for grapefruit, for added PE activity. The juices were then homogenized with a blender for two minutes to insure small, relatively uniform particle size and distribution. The resulting pulpy juice was stirred before packaging samples (30 mL) into sterile polyethylene bags (Fisher Scientific, Pittsburgh, PA) and impulse sealed, retaining as little headspace as possible. Samples were double bagged before being placed in the pressure vessel.

Pressurization

Juice for enzyme analysis was pressurized using a Stansted isostatic high pressure unit (Stansted Fluid Power, Stansted, England) at 600, 700, 800, or 900 MPa for 1, 15 or 30 second dwell time. Runs at 500 MPa were 15 seconds and 1, 5, 15 and 60 minutes. Dwell time is defined as the time spent at the set point pressure.

The packaged 30 mL samples were kept in an ice bath until they were pressurized. The pressure unit was at 5-10 °C before pressurization began. A mixture of ethanol and castor oil (85/15 v/v) constituted the pressure medium. Time to reach the desired pressure was 10-12 seconds while decompression time was approximately 10 seconds. Figure 3 represents a typical pressure profile obtained by the isostatic high pressure unit with a 30 second hold time at 700 MPa. As can be seen from this graphical representation, there were no abnormal spikes or drops in the pressure level. The use of a chiller to cool the pressure vessel jacket and the pressure medium ensured that samples remained in the temperature range of 20-50 °C during processing. All runs were done in duplicate. After pressurization, samples were kept at 0°C until PE activity could be determined.

Figure 4 is a schematic diagram of the Stansted high pressure vessel. The Stansted unit represents the method of direct pressure generation. In direct, or piston type

Figure 3. Typical Pressure Profile Obtained by Stansted Isostatic High Pressure Unit in Rapid Compression/Decompression Mode

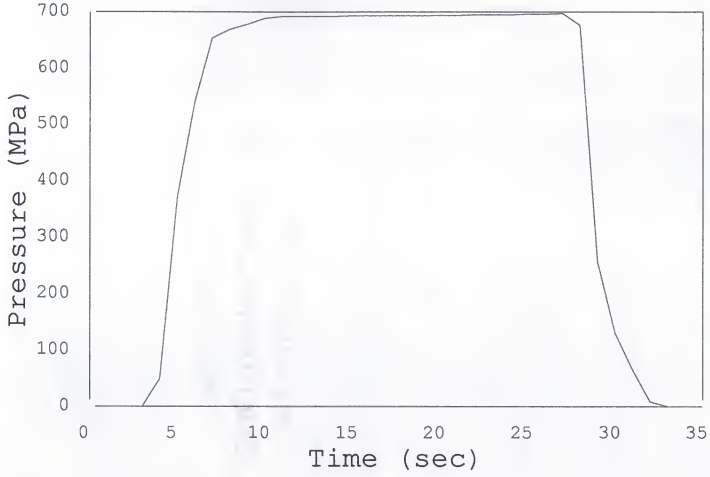

Pressure (MPa)
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
Time (sec)

Figure 4. Stansted "Plunger Press Food Lab 9000" High Pressure Food Processing Unit

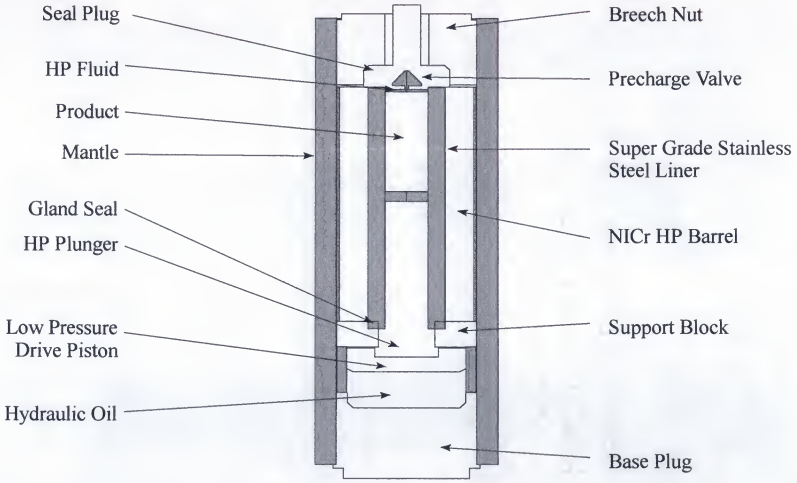

Seal Plug
HP Fluid
Product
Mantle
Gland Seal
HP Plunger
Low Pressure
Drive Piston
Hydraulic Oil
Breech Nut
Precharge Valve
Super Grade Stainless
Steel Liner
NICr HP Barrel
Support Block
Base Plug

Figure 4 is a schematic diagram of the Stansted high pressure vessel. The Stansted unit represents the method of direct pressure generation. In direct, or piston type compression the pressure medium is pressurized directly by the piston, which is driven by a low pressure pump. The hydraulic principle states that the pressure at the large end of the piston is multiplied by the ratio of the two piston sections. This yields the desired high pressure at the small end of the piston. Direct pressure generation is necessary to obtain rapid compression, but is often limited to small diameter pressure vessels because of the limitations of the gland seal (shown in Figure 4).

PE Activity Determination

PE was assayed using the titration method of Rouse and Atkins (1955) using 100 mL of a 1% pectin solution in 1M NaCl. This method is derived from the validated method of Lineweaver and Ballou (1945), and later validated by Seymour et al. (1991) and Warrilow and Jones (1995) The juice/pectin mixture was brought to a pH of 7.5 and titrated with NaOH for at least 10 minutes or 5 mL. Activity is calculated from the equation

mL titrant x N titrant x 10^3 / time x weight of sample EQ. 2

and results were reported as equivalents of enzyme hydrolyzed per minute per gram sample, or pectinesterase

units x 10^3 min^{-1} g^{-1} juice (PEu x 10^3). Pectin from citrus fruits was obtained from Sigma (St. Louis, MO) and had an 8% methoxy content. Pectin solutions were kept at a constant temperature of 28 °C. All samples were titrated in duplicate. Average %RSD of titrations was 8.0 for orange juice and 7.4 for grapefruit.

Enzyme Isolation Study

PE for the detailed inactivation study was obtained from late season Valencia juice extracted with an FMC commercial extractor. Four groups of juice were considered: untreated control, pressurized at 800 MPa for one minute, heated to 70 °C for 1 minute, and heated to 90 °C for one minute. These heating levels were chosen to represent a light pasteurization and a commercial level of pasteurization. After treatment, the juice was centrifuged at 10,000 g for 30 minutes to obtain the pulp used for extraction of the enzyme. Pulp spun out from juice was not washed prior to extraction to prevent resuspension.

Enzyme Extraction

PE was extracted from fresh Valencia pulp via the following method:

1. Weighed sample of pulp was washed with two volumes of deionized water to remove soluble solids.

2. Sample homogenized in blender with 1 L 0.25 M Tris-Cl + 1 M NaCl at pH 8.0 and then stirred at room temperature for 1 hour.

3. Centrifuge at 10,000 x g for 30 minutes, retain supernatant and filter through miracloth (Calbiochem, La Jolla, CA)

4. Slowly add ammonium sulfate to 80% saturation, stirring the whole time. System was kept on ice to retain PE activity. Saturation point was calculated by mL supernatant x 0.7g/mL for 100% saturation. Solution was refrigerated at 4 °C for 12-48 hours to allow protein to precipitate.

5. Solution was centrifuged at 10,000 x g for 30 min under refrigeration temperatures (0°- 4 °C). Pellet was retained and resuspended in a minimum volume of 10 mM sodium phosphate buffer, pH 7.0 adjusted with solid sodium hydroxide.

6. Solubilized pellet was centrifuged for 10 minutes at 10,000 x g to remove insoluble solids. Supernatant was retained, as it contains the desired PE activity.

7. Supernatantwas dialyzed in Spectra-por dialysis membranes against 10mM sodium phosphate buffer with pH 7 from 18 - 24 hours with three buffer changes. System was stirred and kept in a cold room on ice.

The dialyzed supernatant then underwent isoelectric focusing using a Rotofor (Bio-Rad) to separate it into fractions along a pH gradient using ampholytes in the range of pH 8-10.5. Since heat labile PE has an isoelectric point of 9.5 and heat stable PE is suspected to be higher, all fractions with a pH between 9.0 and 11.5 were assayed for PE activity after separation. Fractions were then further separated using SDS-PAGE followed by silver staining.

Sample Preparation

Fractions from isoelectric focusing were dialyzed to remove ampholytes before sample prep for SDS-PAGE. A 7.5 mL volume of sample was combined with 2.5 mL sample buffer. Sample buffer consists of Tris-Cl buffer pH 6.8 with 2% SDS, 10% glycerol and 0.025% Bromphenol Blue. The glycerol provides the density necessary to make the samples sink to the bottom of the wells in the stacking gel. The Bromphenol Blue is a tracking dye to monitor sample progress through the gel during the run.

Cloud Loss

Cloud loss of treated samples was monitored as an indicator of residual PE activity after treatment. Fresh squeezed, mid-season Valencia orange juice from the FMC

extractor in the CREC pilot plant was subjected to pressures from 400 to 900 MPa for 1 second, 1 minute or 10 minutes. The juice was not subjected to a finishing step, and no additional pulp was added. The juice was strained through a U.S. standard #20 mesh screen and then homogenized in the blender on low for 30 seconds. The method for determining cloud loss described by Cameron et al. (1997) involves centrifuging a 50 mL juice sample at 15,000 x g for ten minutes and then measuring the absorbance of the supernatant at 660 nm. Bottles were inverted five times to facilitate mixing before samples (50 mL) were periodically drawn for analysis. Juice bottles were stored at 4 °C between analysis times.

Samples were monitored for microbial growth or contamination by spread plating 0.1 mL juice sample on duplicate orange serum agar (OSA)plates. Dehydrated OSA (Difco)was mixed with water and plates were poured and left 24 hours to set. The plates were incubated at 30 °C for 48 hours before counting colony forming units.

Storage Study of Valencia Orange Juice

Juice was extracted from sound, washed, refrigerated Valencia oranges using an FMC model 291 extractor in the CREC pilot plant. It was finished using an FMC model 35

finisher with a 20 mesh screen. A portion of the juice was immediately canned and frozen for use as the study control. Another portion was packaged in 250 mL portions in a low permeability bag and sealed in a polyethylene bag before being pressurized at 500 MPa for 2 minutes. Samples were stored in an ice bath both prior to and post pressurization until all samples were treated. A third portion of the juice was pasteurized at 95 °C with a 15 second hold time using a Microthermics UHT/HTST Lab, Model 25 (Raleigh, NC) and heat sealed into the above-mentioned bags.

Bags stored at 1.5 °C were monitored for volatile composition at approximately 4 week intervals for a total of 36 weeks. Volatiles were monitored using a Hewlett Packard gas chromatograph, model 5980 series II with flame ionization detector. Sample introduction was accomplished through an OI Analytical (College Station, TX) model 4560 purge-and-trap sample concentrator. An undiluted juice sample (1 mL) was purged for 2 minutes and analyzed on a DB-5 column under the following GC conditions: injector: 225 °C, detector: 250 °C, initial time: 5 minutes, ramp rate 5°/minute from 45 °to 110 °C . Sabinene, myrcene, z-3-hexenol, α-pinene, octanal and d-limonene were monitored for the three different treatments over the course of the study. Tentative identification of peaks was determined by

Kovats indices and comparison to known standards. These compounds were chosen because of their contribution to "fresh orange flavor" (Redd et al, 1996).

Statistics

All statistics presented in this study were computed using Statistica (Statsoft, Tulsa, OK). Principal component analysis and discriminant analysis were performed and all results are reported at the 95% confidence level.

Samples were monitored at approximately 4 week intervals, and duplicates of each sample were run in random order. The values presented represent an average of the peak areas of each separate compound at each sampling time.

RESULTS AND DISCUSSION

Enzyme Inactivation

Fresh Valencia juice has PE activity in the range of 2-6 PEu x 10^3 (Snir et al., 1996). Blending fresh frozen pulp into the sample juices increased juice PE activity to 10-12 PEu x 10^3, the point that at least a log cycle reduction resulting from pressure treatment could be measured by the traditional wet chemical assay. Juice pulp was chosen over prepared enzyme to approximate the natural food system, since Pollard and Kieser (1951) found that enzyme inactivation in a raw juice was distinctly different than the results of a purified enzyme preparation. Also, commercial citrus PE is prepared from citrus peel and may not have the same ratio of isozymes found in the internal parts of the fruit. Since the activity in juice is due mainly to the pulp it was important to use a pectinesterase source that would most closely mirror the typical food system.

Kinetics

Figures 5 and 6 show remaining PE activity versus the dwell time at four different pressures. Inactivation of PE with higher isostatic pressure was bi-phasic, in accordance with the different forms of the enzyme. Prior to this study, two separate slopes of inactivation have been reported for thermal inactivation (Versteeg et al., 1980; Wicker and Temelli, 1988). The first drop in activity after pressurization has been described as an "instantaneous pressure kill" by Basak and Ramaswamy (1996). These researchers investigated pressure effects on PE in the range of 100 to 400 MPa, and observed a much less pronounced initial drop than is seen in Figures 5 and 6 which illustrate higher pressures and shorter compression time. The time to reach the set point (come-up time) was longer on their pressure equipment, taking up to 3 minutes. They pointed out the come-up time at the lower pressures should not have much effect. Dwell times in their study were as long as 720 minutes. Since a hold time of this duration is commercially impractical, the need for information at higher pressures is clearly indicated.

Seyderhelm et al. (1996) reported the effect of higher pressures on PE, but the data given was for commercial PE in pH 7 tris buffer at 45 °C. The shortest processing time

Figure 5. Inactivation of Orange Pectinesterase at Pressures of 600-900 MPa. Data Points Represent the Average of Two Measurements

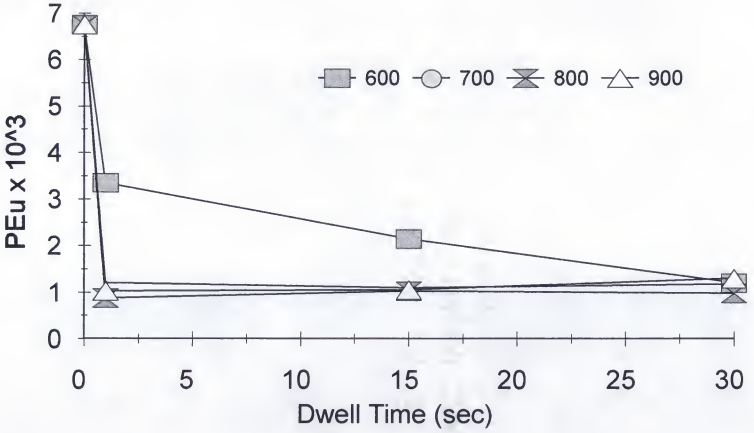
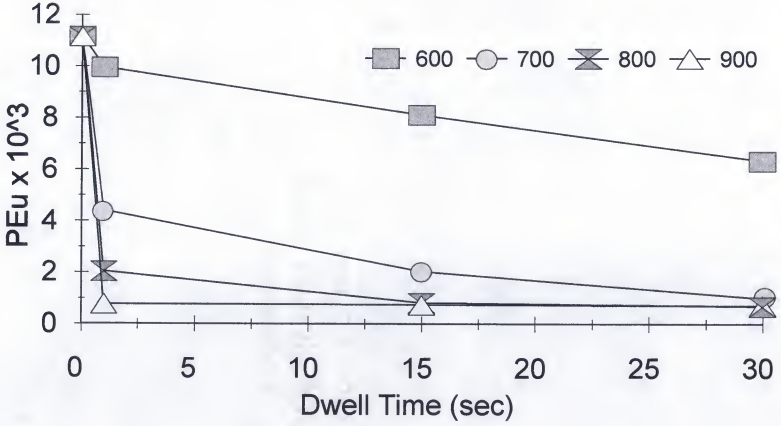

600
700
800
900
PEu x 10^3
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30
Dwell Time (sec)

Figure 6. Inactivation of Grapefruit Pectinesterase at Pressures of 600-900 MPa. Data Points Represent the Average of Two Measurements


12
10
8
6
4
2
0
PEu x 10^3
600
700
800
900
0
5
10
15
20
25
30
Dwell Time (sec)

shown, 2 minutes, was sufficient to completely inactivate PE at 900 MPa. An approximately 45 °C increase in temperature can be expected at 900 MPa (Morild, 1992), so it is possible that the complete inactivation was augmented by heat. At 600, 700 and 800 MPa, less inactivation of PE was experienced in buffer (Seyderhelm et al. 1996) compared to the 15 second results in orange juice (Figure 5). Although data on enzymes isolated from their native environment are important, this discrepancy stresses the need for empirical data using the natural enzyme in the appropriate biochemical model to assess applicability to real food systems.

It was suspected that the initial drop in activity was due to inactivation of heat labile PE, while the remaining activity illustrated the effect of pressure on the heat stable PE (Figs 5 & 6) (Irwe and Olsson, 1994). The heat labile PE comprises from 86 - 94% of the total enzyme in Valencia juice (Snir et al., 1996), and at higher pressures the rapid inactivation is very close to this percentage.

Sun and Wicker (1996) confirmed that exposing juice to pH extremes (pH 2 for 5 minutes) also can inactivate the heat labile form, but this treatment was ineffective against the heat stable form. Subjecting the orange and grapefruit juice to a pH of 2 for five minutes caused 91% inactivation of total PE activity in orange juice. The residual PE activity following higher pressure treatments of juice was

similar to the activities reported after low pH treatment, which suggests that the remaining activity represented heat stable PE.

Table 1 is a summary of the inactivation percentages for orange and grapefruit juice at varying pressures and acid treatments. Subsequent heating of a pressurized (1 minute at 700 MPa) orange juice sample for 2 minutes at 70 °C did not reduce PE activity, while heating for 2 minutes at 90 °C resulted in a marked decline in PE activity, from 0.2 to 0.1 PEu x 10^3, substantiating that only the heat stable form remained after pressurization. These temperatures were chosen because they represent two levels of heating that can distinguish the two isozymes (Versteeg et al. 1980).

Heat Generation by Pressure

The question of whether or not the heat generated by pressurization was sufficient to inactivate PE was considered. Samples were placed in the unit at 5°- 10 °C and reached temperatures between 20 °and 50 °C (measured by a thermocouple) depending on set point pressure. Immediate cooling occurred upon decompression. Morild (1992) described the temperature change due to pressure changes as $\alpha TV/C_p = 1.86 \times 10^{-3}$ K bar^{-3}. The pressure medium used in this study was 15% castor oil, which has a heat capacity

Table 1 . Percent Inactivation of Pectinesterase in Orange and Grapefruit Juice at Different Pressures

Treatment (1 second)	Orange	Grapefruit
600 MPa	10	50
700 MPa	61	82
800 MPa	82	87
900 MPa	93	85

(C_p) of 2.1 J/g °C and 85% ethanol. The heat capacity of pure ethanol is 1.4 J/g °C. Ethanol was 5% water,, so the heat capacity of the ethanol component was 1.5 J/g °C. A combination of these values at the appropriate ratio results in a heat capacity of the pressure medium equal to 1.6 J/g °C. Substituting this value of C_p for that of water in the equation results in a conversion of 4.8×10^{-3} K/bar. One bar is equal to 0.1 MPa, so after adjusting the equation to the heat capacity of the pressure medium and converting to MPa, the conversion factor becomes 4.8×10^{-2} K MPa^{-1}. At the highest pressure used in this study, 900 MPa, the maximum theoretical temperature increase is 43.2 °C. Again, the heating will not be adiabatic because of heat exchange with the chilled vessel wall. This confirms that temperatures generated by pressures used in this study were not sufficient to thermally inactivate PE.

Figure 7 shows pressure treatment at 500 MPa, illustrating the difference in curve shape between lower (<600 MPa) and higher (600-900 MPa) pressures. At <600 MPa, it is possible to observe the first order inactivation of heat labile PE. A plot of the log of the data from 500 MPa (Figure 8) gives a regression equation of

$$y = (-1.9 \times 10^{4})x + 1.08 \qquad \text{EQ. 3}$$

The slope can be used to determine the decimal reduction value, D_p and the inactivation coefficient, k. D_p and k

Figure 7. High Pressure Inactivation of Pectinesterase at 500 MPa. Data Points Represent the Average of Two Measurements

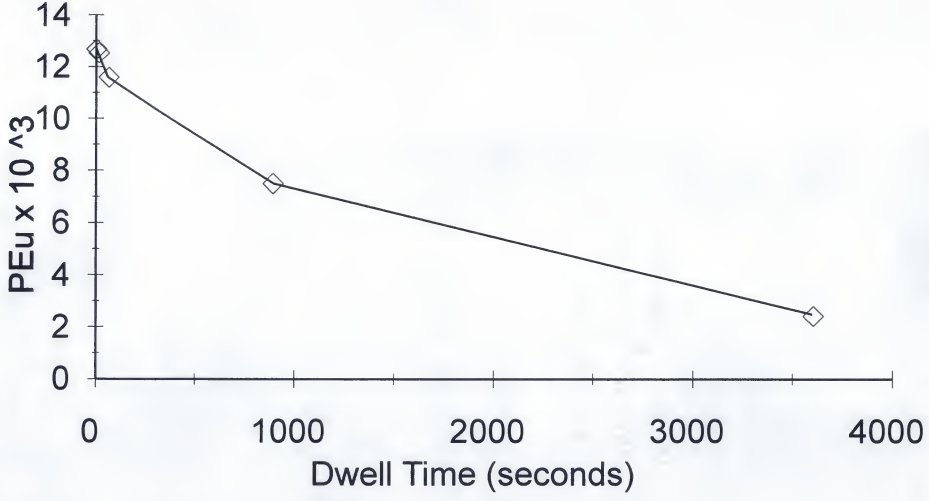
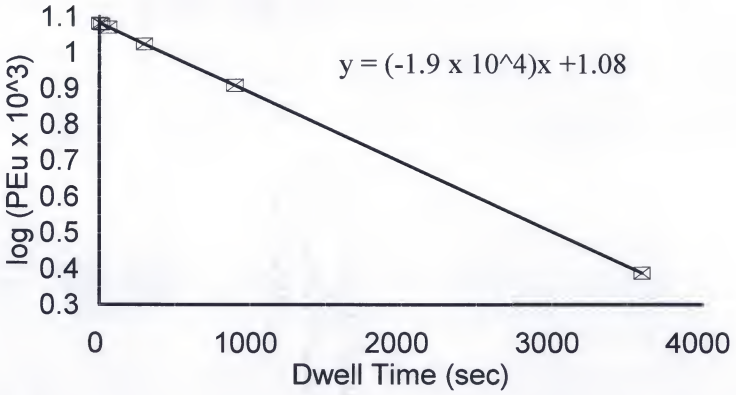

14
12
10
8
6
4
2
0
PEu x 10 ^3
0
1000
2000
3000
4000
Dwell Time (seconds)

Figure 8. Log of PE Inactivation at 500 MPa


y = (-1.9 x 10^4)x +1.08
log (PEu x 10^3)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
1000
2000
3000
4000
Dwell Time (sec)

values will be discussed later. At 700 MPa and above, pressure application inactivates this fraction more rapidly than the 1 second minimum dwell limitation of the equipment, leaving the heat stable form active (see Figures 5 and 6). The time required to reach the set point pressure was approximately 10 seconds, so the enzyme spent some time at the lower pressures before starting the dwell time counter, contributing to the inactivation of the enzyme. Higher pressures inactivate the heat labile form too quickly to measure this decline.

Longer processing times at >600 MPa did not inactivate the remaining heat stable form. This result, coupled with the observation that pectinesterase does not recover from high pressure treatment (Ogawa et al., 1992) puts this enzyme in the group that is incompletely and irreversibly inactivated (Miyagawa et al., 1964). The existence of a maximum pressure above which no extra inactivation is apparent has also been observed in trypsin, chymotrypsin and chymotrypsinogen (Curl and Jansen, 1950a &b). Samples held at 700 and 800 MPa for as long as 1 minute had little decrease in activity over samples held for a 15 second dwell time. These results showed that dwell times of 15 seconds or less were sufficient to reduce PE activity in orange juice, with inactivation increasing significantly with increasing pressures in both juices.

Analysis of variance (ANOVA) designated the probability of difference between PE inactivation and pressure levels as 100% for both juices. Varying dwell times at the higher pressures did not cause significantly different PE inactivation in grapefruit juice (80% probability of difference, p=.193) but caused significant differences in orange juice PE at a probability level of 100% (p=0.000).

Decimal Reduction Values

The time necessary to reduce activity one log cycle, or 90% at a given pressure, is defined as the D_p value. The D_p value of PE in orange juice for 600 MPa was 143 seconds (2.4 min), while the D_p value for 500 MPa was 5000 seconds (83.3 minutes). The slope of the regression line of 500 MPa adjusted by 2.303 for the natural log yields a k of 4.4 x 10^{-4} s^{-1}, while the k value at 600 MPa is 7.5 x 10^{-3} s^{-1}. Not surprisingly, the enzyme is more rapidly inactivated at 600 MPa than at 500 MPa. This represents inactivation of the heat (and pressure) labile forms of PE only. Since the enzyme has been shown to be biphasic, reporting one decimal reduction value for both forms is inaccurate. No inactivation of the heat stable form was noted, thus no inactivation kinetics are reported.

The time or pressure necessary to cause a 90% reduction in the D value is termed the z value. The z_p value

corresponding to the 500-600 MPa range was 65 MPa. Basak and Ramaswamy (1996) report a D_p value of 260 minutes at pH 3.7 and 14 minutes at pH 3.2 at 400 MPa. The juice used in this study was in the middle of this pH range at 3.45. For comparison, the temperature necessary to accomplish 90% PE inactivation in orange juice in less than a minute was reported as 85°C by Rouse and Atkins (1952).

Enzyme Sensitivity

Comparison of Figures 5 and 6 shows that grapefruit PE was initially more sensitive to pressure treatment than orange. The same observation was made for the sensitivity of grapefruit PE to thermal inactivation (Rouse and Atkins, 1952). Due to the high percentage of grapefruit PE rapidly inactivated at 600 MPa, no D_p value was calculable from the pressure data of this study. Table 1 shows that grapefruit PE inactivation is not greater than ~ 85% even at the highest pressures used. Comparing this to the values presented for orange, one may initially form a contrary conclusion about the sensitivity of grapefruit PE, but it is hypothesized that since grapefruit PE has a percentage of heat stable enzyme as high as 33% (Rombouts et al., 1982), it will not experience as much total inactivation as orange PE by high pressure.

It is proposed that the heat labile form of PE in grapefruit was more sensitive to pressure treatment. To substantiate the assertion that only the heat stable form remained after high pressure treatment, pressurized grapefruit juice samples (700 MPa for 1 minute) were heated to either 70 °C or 90 °C and then assayed for PE activity after the sample was cooled to 4 °C. The 70 °C treated sample showed no change in activity, while the 90 °C sample decreased in PE activity from 1.5 PEu to 0, showing complete inactivation of both forms.

pH Effects

A major contributor to this sensitivity is the fact that grapefruit juice is of a lower pH than orange, sometimes as much as 0.5 pH units. It has already been mentioned that enzyme inactivation takes place more readily in a lower pH solution when either heat or high pressure is the method of inactivation. This fact is thoroughly established in citrus juices by the work of Rouse and Atkins (1952, 1953). Their results showed that PE was inactivated at lower temperatures if the starting juice was at a lower pH.

Enzyme Isolation

Enzyme isolation and separation was performed to compare the effects of heat and pressure treatment on PE isolated from pulp. Figures 9-12 show the results of the SDS-PAGE separation of the IEF fractions of PE isolated from fresh and treated Valencia orange juice. Figure 9 represents the control, which has a tight band at 36,000 Dalton, typical of PE (Seymour et al.,1991). Figure 12 represents the juice treated at 90 °C, approximating commercial pasteurization. The absence of a protein band at 36,000 D in the gel in Figure 12 is an indication that the pectinesterase enzyme protein has been denatured and precipitated before analysis by electrophoresis. This is confirmed by the absence of any measurable PE activity in the 90 °C heated sample.

The figures (9 & 10) depicting the sample pressurized at 800 MPa for 1 minute and the juice heated to 75 °C show very similar results by gel electrophoresis. There is an ill resolved band around 36,000 molecular weight indicating that although pectinesterase is present, it is different from the control. The possibility of sample overloading was considered but ruled out, as protein concentration was similar in all samples. Both the pressurized and the 75 °C samples show a decrease in total activity over the control.

Figure 9. SDS-PAGE Separation of Pectinesterase from Untreated Valencia Juice
Lane 1= Mid Range Ladder, 2=pH 9.1, 3=pH 9.35, 4=pH 9.62, 5=pH 9.95, 6=pH 10.81, 7=pH 11.59, 8-10= raw extract

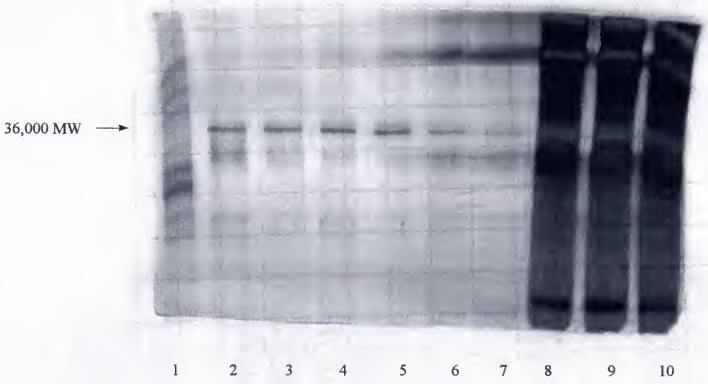

36,000 MW
1
2
3
4
5
6
7
8
9
10

Figure 10. SDS-PAGE Separation of Pectinesterase from Valencia Juice Pressurized at 800 MPa for 1 Minute. Lane 1=pH 9.16, 2=Mid Range Ladder, 3=pH 9.3, 4=9.41, 5=pH 9.55, 6=pH 9.72, 7=pH 10.01, 8=pH 10.32 9=pH 11.06, 10=pH 11.96

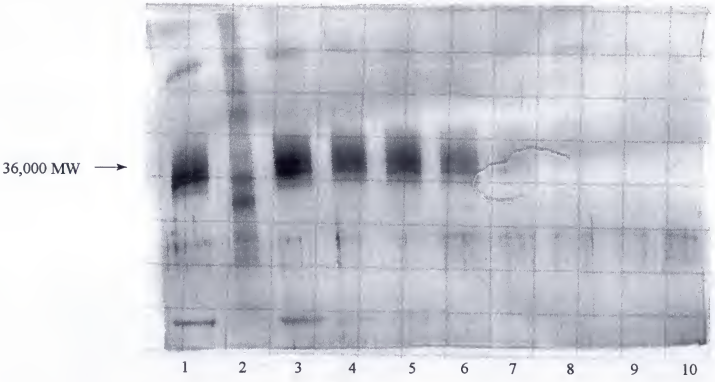

36,000 MW
1
2
3
4
5
6
7
8
9
10

Figure 11. SDS-PAGE Separation of Pectinesterase from Valencia Juice Pasteurized at 75 °C for 1 Minute. Lane 1=Mid Range Ladder 2=pH 9.15, 3=pH 9.37, 4=pH 9.52, 5=pH 9.67, 6=pH 9.89, 7=pH 10.89, 8=pH 10.33 9=pH 11.15 10=pH 12.52

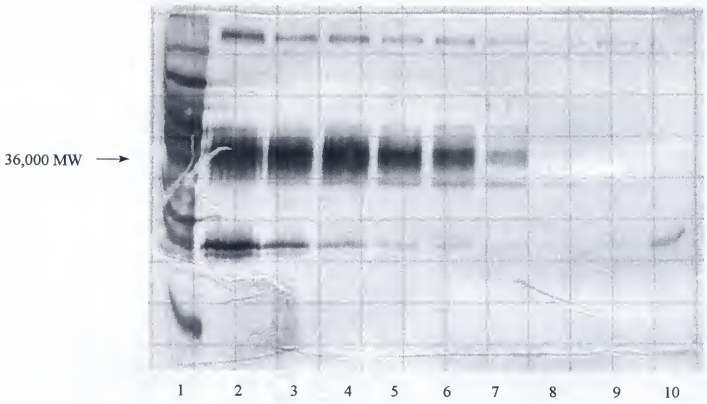
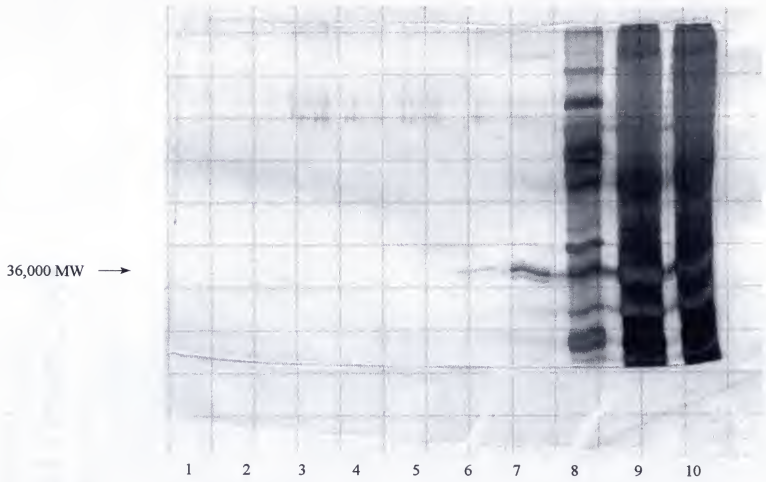

36,000 MW
1
2
3
4
5
6
7
8
9
10

Figure 12. SDS-PAGE Separation of Pectinesterase from Valencia Juice Pasteurized at 90 °C for 1 Minute. Lane 1=pH 9.07, 2=pH 9.30 3=pH 9.52, 4=pH 9.50, 5=pH 9.84, 6=pH 11.09, 7=pH 11.71, 8=Mid Range Ladder, 9-10= Raw Extract


36,000 MW
1
2
3
4
5
6
7
8
9
10

Tables 2-4 list activities of the control and the differing treatments as well as the pH of the fractions from isoelectric focusing. It is important to compare fractions with similar pH, because the protein will be found in the pH range that brackets its pI. For PE this is 9.5, and consideration of Tables 2-4 illustrates that these pH values are indeed those with the highest specific activity. As expected, the control had the highest pectinesterase activity.

The 75 °C and pressure treatments were expected to inactivate approximately 90% of the total PE activity as was seen with extracted pressurized pulp, but this was not the case. Since the specific activity is reported per mg of total protein in the IEF fraction it is possible that some other contaminating protein is present in the control, which would make the reported specific activity lower.

Several theories exist regarding the existence of heat labile and heat stable forms of PE. Some believe the heat stable form to be at a higher molecular weight than the 36,000D of the heat labile form. Other evidence points to the possibility that both forms have the same molecular weight, but differ in their ability to tolerate heat. The heat stable form of PE has a higher percentage of hydrophobic amino acids than the heat labile fraction (Seymour et al., 1991), which could account for its greater

Table 2. Specific Activity of Isoelectric Focussed Fractions of Pectinesterase Isolated from Untreated Late Season Valencia Orange Juice

Fraction	Specific Activity (PEu/mg protein)	pH
15	0.024	9.1
16	0.068	9.35
17	0.071	9.62
18	0.036	9.95
19	0.008	10.81

Table 3. Specific Activity of Isoelectric Focussed Fractions of Pectinesterase Isolated from Late Season Valencia Orange Juice Treated at 75 °C for 1 Minute

Fraction	Specific Activity (PEu/mg)	pH
13	0.059	9.52
14	0.047	9.67
15	0.045	9.89
16	0.033	10.89
18	0.038	11.15

Table 4. Specific Activity of Isoelectric Focussed Fractions of Pectinesterase Isolated from Late Season Valencia Orange Juice Treated by High Pressure (800 MPa) for 1 Minute

Fraction	Specific Activity (PEu/mg)	pH
14	0.048	9.41
15	0.046	9.55
16	0.039	9.72
17	0.037	10.01
18	0.033	10.32

resistance to heat and pressure denaturation. Higher hydrophobic amino acid content in an aqueous ionic system (citrus juice) will result in unfavorable solvent interactions causing the sphere of hydration of the denatured enzyme to be greater than the native form. Thus, denaturation will be more thermodynamically unfavorable in the heat stable form because of solvent interaction and an increase in hydration volume. In addition, the increase in hydration volume will, according to LeChatelier's principle, be restricted by the application of high pressure.

Cloud Loss

Figures 13-17 illustrate the effectiveness of HPP in preserving cloud in orange juice. Five different pressure levels were employed and the cloud loss over time was compared to cloud loss in an untreated control. Treatment at 500 MPa (Fig 13) did not yield any significantly greater stability over the untreated control, although a 10 minute hold time was able to increase shelf life by two weeks. Treatment at 600 MPa (Fig 14) had better results, with 10 minute treatment yielding a cloud stable product for the duration of the 90 day study. As might be expected, shorter treatment times were less effective, but a 1 minute processing time afforded much greater stability over the

Figure 13. Cloud Stability of Orange Juice Treated at 500 MPa (1 sec,■; 1 min, ●; 10 min, ▲; control,▼)

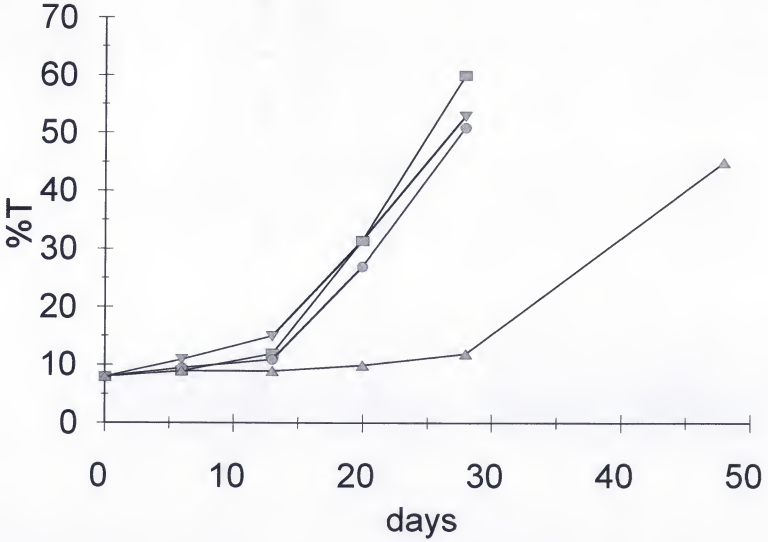

70
60
50
40
30
20
10
0
%T
0
10
20
30
40
50
days

Figure 14. Cloud Stability of Orange Juice Treated at 600 MPa (1 sec,■; 1 min, ●; 10 min, ▲; control,▼)

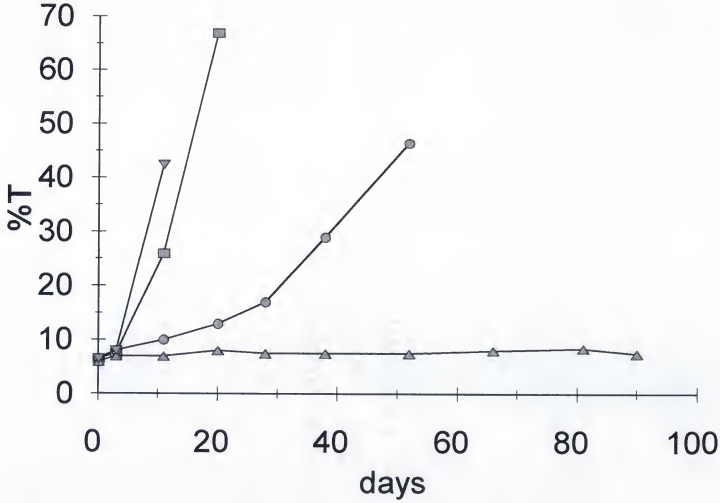

70
60
50
40
30
20
10
0
%T
0
20
40
60
80
100
days

Figure 15. Cloud Stability of Orange Juice Treated at 700 MPa (1 sec,■; 1 min, ●; 10 min, ▲; control,▼)

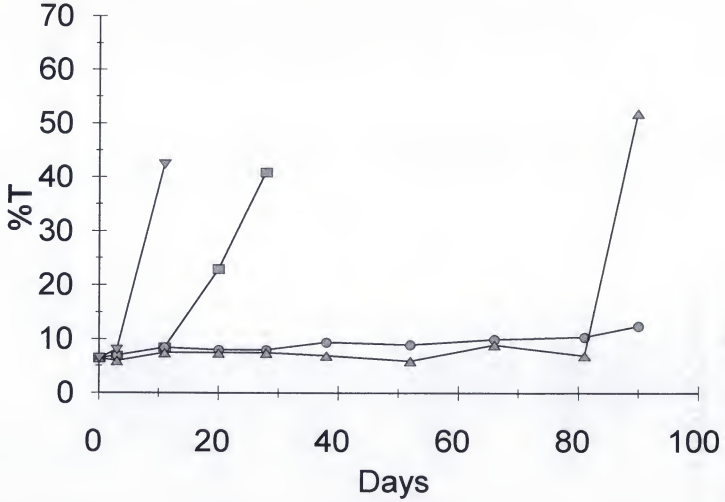

70
60
50
40
30
20
10
0
%T
0
20
40
60
80
100
Days

Figure 16. Cloud Stability of Orange Juice Treated at 800 MPa (1 sec,■; 1 min, ●; 10 min, ▲; control,▼)

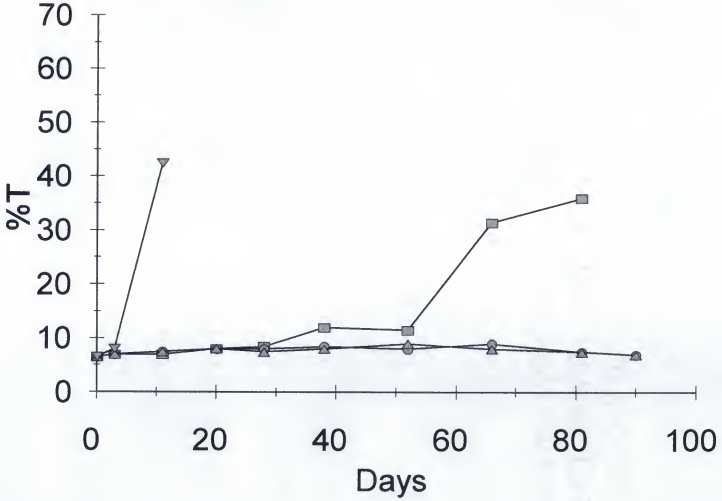
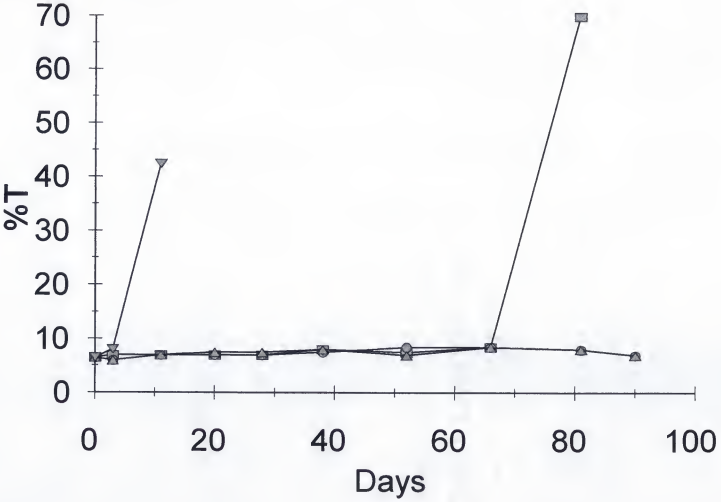

70
60
50
40
30
20
10
0
%T
0
20
40
60
80
100
Days

Figure 17. Cloud Stability of Orange Juice Treated at 900 MPa (1 sec,■; 1 min, ●; 10 min, ▲; control,▼)


70
60
50
40
30
20
10
0
%T
0
20
40
60
80
100
Days

control and extended cloud stability to 49 days. At 700 MPa, treatment by high pressure is much more effective, as a 1 minute treatment at 700 MPa stabilized cloud for the full 90 day duration of the study. Samples treated for 10 minutes were stable until the last week of the study, when they completely lost all cloud. These samples were found to be microbially stable and no explanation was discovered. Only molds are able to clarify juice through the production of extracellular enzymes such as pectinesterase (Nussinovitch and Rosen, 1989), so absence of contamination by a yeast or bacteria would not be unusual, as was the case in this sample. It is possible that mold growth was present but not detected by plating. Visible mycelia in the sample was absent and is not necessary to accomplish cloud loss or clarification of the sample. Cloud was monitored for 90 days because this point signifies flavor deterioration in commercial, packaged, not-from-concentrate refrigerated orange juice.

Pressures of 800-900 MPa (Figs 16-17) were much more successful in preserving cloud at shorter processing times. One second treatment at these higher pressures was effective at preserving cloud for up to 80 days. Longer processing times of 1 or 10 minutes maintained a cloud level that was unchanged from the initial cloud content of the juice at time zero. Thus, the samples at 800 and 900 MPa at

processing times of 1 minute or above are considered to suffer no appreciable cloud loss at refrigerated storage (4 °C) over a period of 90 days.

Table 5 summarizes plate counts taken during the cloud loss study. A dashed line in the table indicates that a particular sample was not cloud stable at the time of microbiological sampling, so that particular juice sample was eliminated from microbial analysis. The two samples in Table 5 listed as "TNTC" were most likely contaminated post-processing by *Rhodotorula* yeast, which has not been shown to affect cloud in citrus juice. *Rhodotorula* was identified by its characteristic pink color and examination under a microscope to determine cell size was that of yeast and not bacteria.

Storage Study

Statistical Analysis of Flavor Volatiles

Figures 18 to 23 are the peak areas of the six monitored compounds used in this study versus the storage time in weeks. Figure 18 (z-3-hexenol) has little interpretive merit as there are no obvious trends or patterns and the treatments are not well separated from each other. Figure 19 (α-pinene) shows a definite difference among the three treatments. There are three distinct levels of α-pinene corresponding to the three treatments. The

Table 5. Plate counts on OSA at days 49 and 89 of cloud loss study. Counts are reported using standard plate counting protocol

Treatment Time	600 MPa	700 MPa	800 MPa	900 MPa
1 second(49)	-------	--------	40 est.	TNTC
1 minute(49)	145 est.	<10 cfu/mL	10 est.	15 est.
10 minutes(49)	50 est.	<10 cfu/mL	75 est.	5 est.
1 second(89)	-------	--------	5 est.	--------
1 minute(89)	-------	15 est.	5 est.	145 est.
10 minutes(89)	<10 cfu/mL	5 est.	5 est.	TNTC

Figure 18. z-3-hexenol Composition Over Storage Time
Treatments: c=Control, p=Pressurized, h= Heat Pasteurized

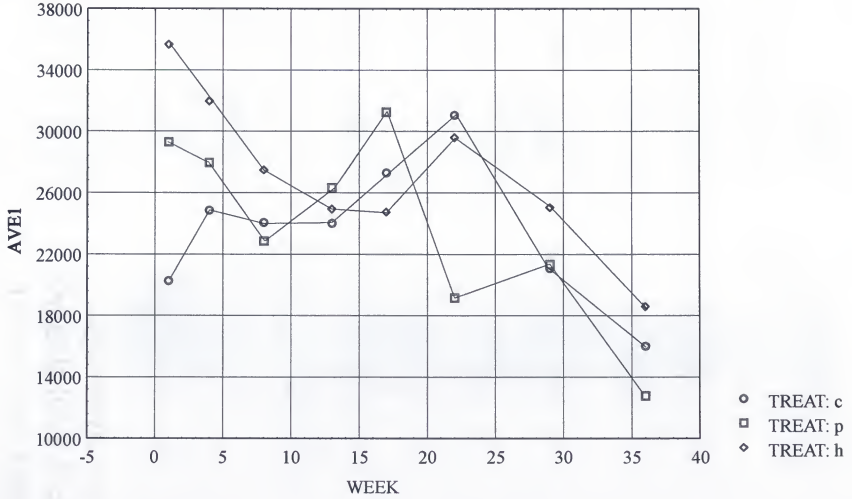

38000
34000
30000
26000
22000
18000
14000
10000
AVE1
-5
0
5
10
15
20
25
30
35
40
WEEK
TREAT: c
TREAT: p
TREAT: h

Figure 19. α-pinene Composition Over Storage Time
Treatments: c=Control, p=Pressurized, h= Heat Pasteurized

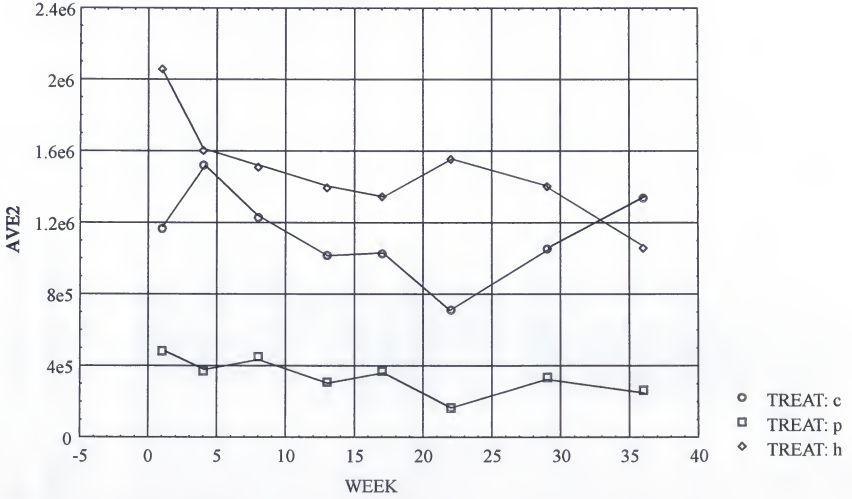

2.4e6
2e6
1.6e6
1.2e6
8e5
4e5
0
AVE2
-5
0
5
10
15
20
25
30
35
40
WEEK
TREAT: c
TREAT: p
TREAT: h

Figure 20. Sabinene Composition Over Storage Time
Treatments: c=Control, p=Pressurized, h= Heat Pasteurized

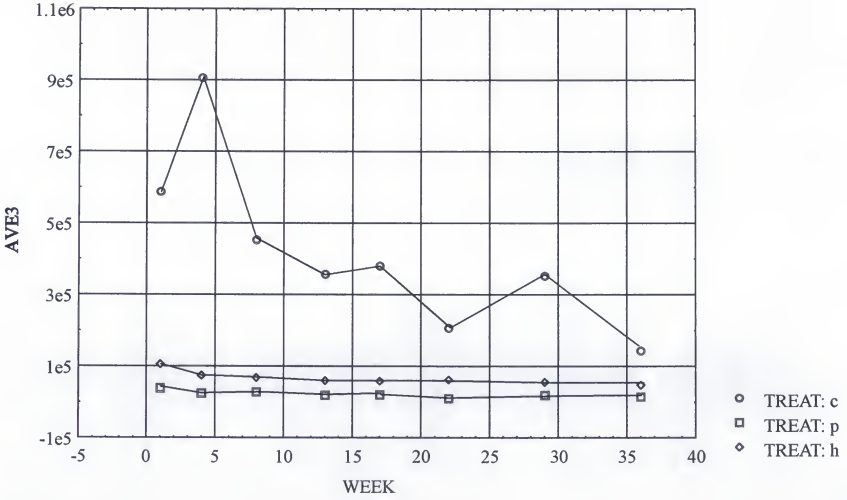

1.1e6
9e5
7e5
5e5
3e5
1e5
-1e5
AVE3
-5
0
5
10
15
20
25
30
35
40
WEEK
TREAT: c
TREAT: p
TREAT: h

Figure 21. Myrcene Composition Over Storage Time
Treatments: c=Control, p=Pressurized, h= Heat Pasteurized

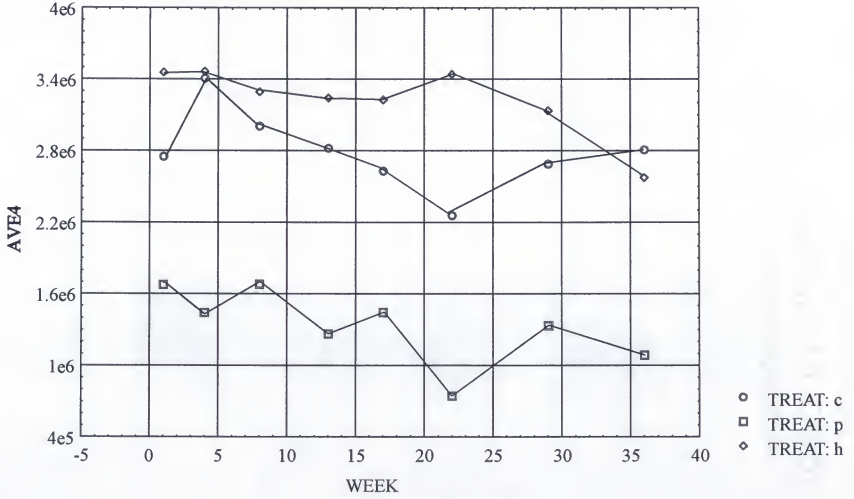

4e6
3.4e6
2.8e6
2.2e6
1.6e6
1e6
4e5
AVE4
-5
0
5
10
15
20
25
30
35
40
WEEK
TREAT: c
TREAT: p
TREAT: h

Figure 22. Octanal Composition Over Storage Time
Treatments: c=Control, p=Pressurized, h= Heat Pasteurized

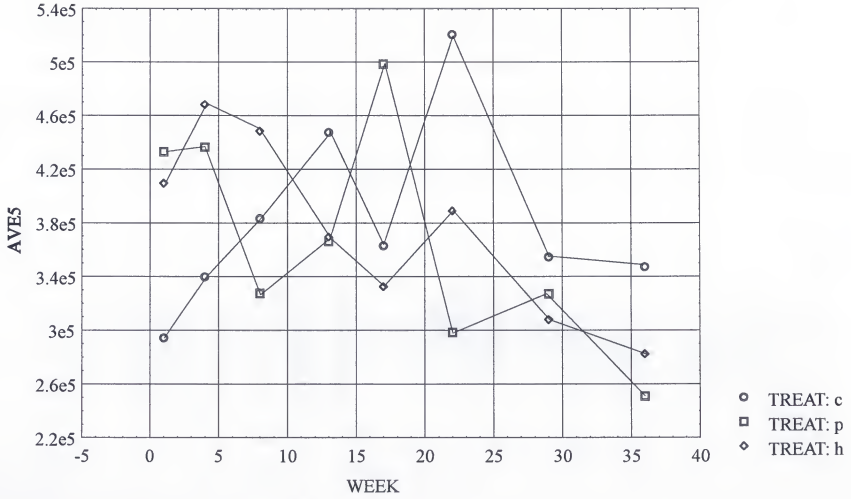
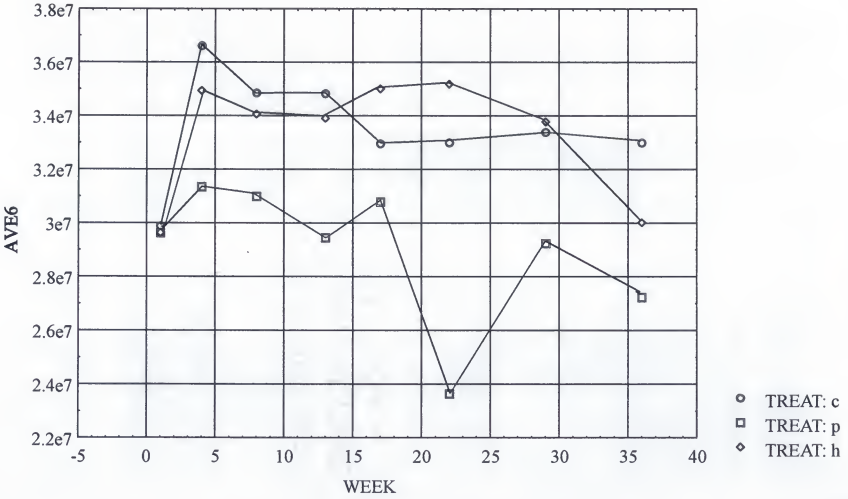

5.4e5
5e5
4.6e5
4.2e5
3.8e5
3.4e5
3e5
2.6e5
2.2e5
AVE5
-5
0
5
10
15
20
25
30
35
40
WEEK
TREAT: c
TREAT: p
TREAT: h

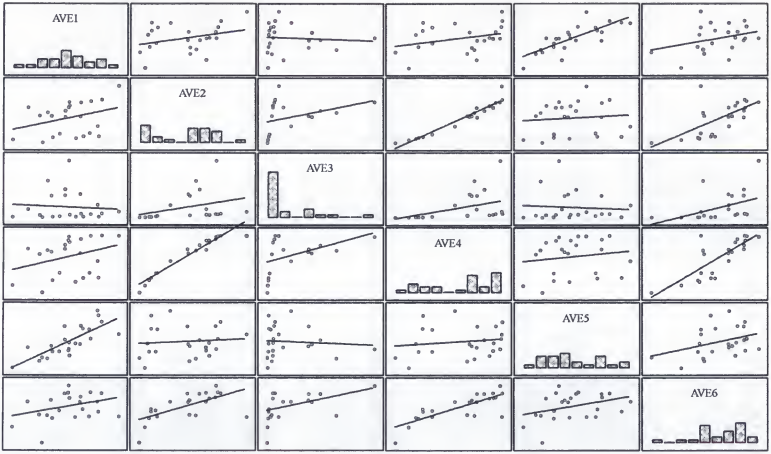
Figure 23. d-Limonene Composition Over Storage Time
Treatments: c=Control, p=Pressurized, h= Heat Pasteurized


3.8e7
3.6e7
3.4e7
3.2e7
3e7
2.8e7
2.6e7
2.4e7
2.2e7
AVE6
-5
0
5
10
15
20
25
30
35
40
WEEK
TREAT: c
TREAT: p
TREAT: h

pasteurized samples have the largest peak areas while the pressurized have the lowest. There are no apparent trends with regard to the storage time. Figure 20 (sabinene) has a clear separation between the control and the treated samples. The treated samples have very similar levels of sabinene and the level is fairly consistent throughout the storage study. Figure 21 shows myrcene to be consistently lower in the pressure treated sample over time as compared to the control and heat pasteurized juice. Clearly, octanal values (Figure 22)poorly of discriminate among treatment types. All three treatments are intermingled and no clear trends are discernable for any treatment. Figure 23 illustrates the effect of storage on levels of d-limonene. Again, the pressurized samples appear to be separate from the other two treatments, but the control and the heat pasteurized samples are not clearly separated.

Figure 24 is a compilation of the correlations of the six peaks with each other. The x-axis is determined by the column where the graph appears while the y-axis is determined by the row. For example, the graph in the lower left shows the correlation between peak 1 (x-axis) and peak 6 (y-axis). Similarly, the graph in the upper right shows the correlation between peak 6 (x-axis) and peak 1 (y-axis). Upon close inspection of the mini-graphs in Figure 24 , it

Figure 24. Compilation of Monitored Peak Correlation


AVE1
AVE2
AVE3
AVE4
AVE5
AVE6

can be seen that peaks 2 and 4, peaks 2 and 6, peaks 4 and 6, and peaks 1 and 5 are positively correlated.

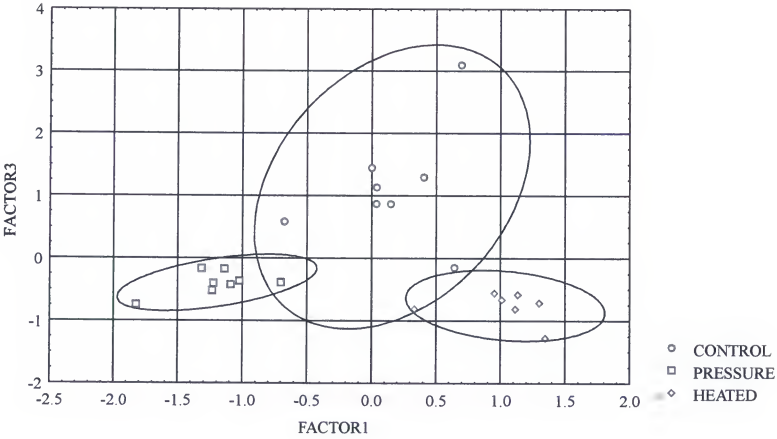
Principal Component Analysis

Principal component analysis is a statistical technique that is able to represent an n-dimensional set of data in a smaller number of dimensions. The basic premise is to reduce the number of variables by forming a linear combination of variables and expressing it as one factor. In this work, the six peaks monitored over time comprise the different variables combined to determine if the samples can be separated into classes or groups based on the treatment they received prior to storage. Figure 25 represents the results of the volatiles monitored for the control and the two different treatments made to the juice after performing principal component analysis and plotting factor 1 versus factor 3. Here, a 6 dimensional set of data has been reduced to 2 dimensions. Extraction of principal components is accomplished by rotating the axes so that the data have maximum variance. This process is best represented by the equation for a new variable

$$factor_1 = ax_1 + bx_2 + cx_3 + dx_4 + ex_5 + fx_6 \qquad \text{EQ. 4}$$

where $x_1..x_6$ are the six variables and a..f are determined by the PCA algorithm and are called factor loadings. From this plot of linear combinations of variables, clusters of

Figure 25. Principle Component Analysis of 6 Volatiles During Storage Study Utilizing Factor 1 and Factor 3. Ovals Represent 95% Confidence Limits


4
3
2
1
0
-1
-2
FACTOR3
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
FACTOR1
CONTROL
PRESSURE
HEATED

samples as well as outliers are discernable. It can be seen in Figure 25 that the pressurized samples and the pasteurized samples form distinct and separate clusters of data points, indicating that the treatments coupled with storage produced distinctive patterns in the measured components. The control samples do not form as tight a group of data. There is one data point that is near the pressurized data, one that is near the heat treated data, and one that is near neither set of data. The remaining five data points are tightly clustered and are approximately equidistant from the three outliers. It is clear from Figure 25 that principal component analysis was able to show that the data formed characteristic groupings based solely on the treatment they received.

Table 6 is a list of the factor loadings used in producing Figure 25. Since Statistica uses correlation matrices in computing the factor loadings in principal components analysis, the factor loadings may be used to note correlations. The factor loadings that are in bold typeface are all heavily weighted. This indicates that they are highly correlated. Peaks 2, 4 and 6 in factor one are correlated while peaks 1 and 5 in factor two show the highest correlation. This is the same information that is gathered from Figure 24 through consideration of the appropriate mini-graphs.

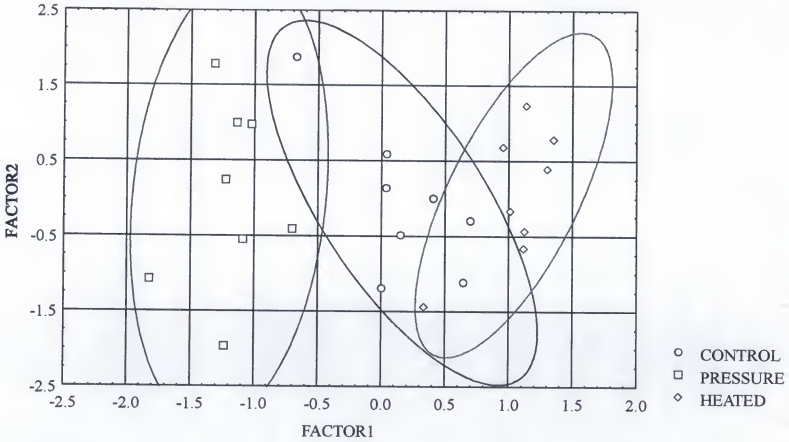
Table 6. Factor Analysis for Principal Component Analysis

Factor Analysis	Factor Loadings		
Variable	Factor 1	Factor 2	Factor 3
z-3-hexenol	0.250325	**0.892601**	-0.111172
α-pinene	**0.969869**	0.065030	0.078037
sabinene	0.234320	-0.093061	**0.945647**
myrcene	**0.971609**	0.110302	0.190194
octanal	0.024991	**0.964449**	0.31959
d-limonene	**0.723407**	0.316738	0.402112
Experimental Variance	2.526179	1.852277	1.111585
Proportion Total (%)	42.1	30.9	18.5

At the bottom of Table 6 are the proportion of the total variance that is represented by the factors. Factor one represents 42.1% of the total variance, factor two represents 30.9%, and factor three represents 18.5%. This means that 91.5% of the total variance of the six variables can be displayed by using only 3 variables. Figure 25, mentioned above, shows factor 1 vs. factor 3 which represents 60.6% of the total variance. In a similar manner, Figure 26 is a graph of factor 1 vs. factor 2 and represents 73% of the variance. Factor 1 (peaks 2,4, and 6) versus factor 2 (peaks 1 and 5) shows some considerable overlap of the heated and control treatments. This indicates that while peaks 1 and 5 have a lot of variance they are not conducive to separating the treatments into discernable groups. One can surmise from Figure 25 that factor 3 is more useful in separating the treatments than factor 2 for separating the groups. Therefore, since factor 3 is mainly comprised of peak 3, peak 3 is better than peaks 1 and 5 at grouping the data based on the treatment the samples received. In short, α-pinene, sabinene, myrcene and d-limonene were useful indicators of treatments while z-3-hexenol and octanal were not.

Factor 1 versus factor 3 is better at separating the data into groups. Examination of the loadings in Table 6

Figure 26. Principle Component Analysis of 6 Volatiles During Storage Study Utilizing Factor 1 and Factor 2. Ovals Represent 95% Confidence Limits

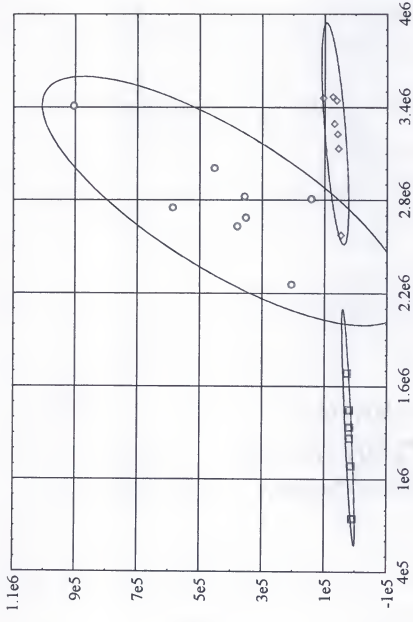

2.5
1.5
0.5
-0.5
-1.5
-2.5
FACTOR2
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
FACTOR1
CONTROL
PRESSURE
HEATED

shows the most heavily weighted compound in factor 1 is peak 4 and peak 3 in factor 3. Therefore, peaks 4 and 3 (Figure 27) were chosen to represent an estimate of the statistical analysis presented in Figure 25. Comparing these two figures it can be seen that only two peaks are necessary to provide an excellent separation of the data based on treatment. Hence, PCA has been successful in reducing the dimensionality of the data. This is a perfect example of how PCA can be used to successfully reduce the complexity of a problem to something manageable.

Discriminant Analysis

The general purpose of discriminant analysis is to determine which variables in a given set of data can be used to discriminate among the different groups. The basic idea behind discriminant analysis is to use the mean of a variable to determine if groups differ, and then use those variables to predict to which group (if any) a new unknown sample value would belong. Here, discriminant analysis uses the average peak area of a control or treated juice sample during the course of storage to determine boundaries that will discriminate and/or predict which treatment a sample received. The variables used in this study are the average of two peak areas of six volatiles monitored by purge and trap GC. The peaks monitored were as follows: peak 1= z-3-

Figure 27. Discrimination Among Treatments Based on Peak Areas of Sabinene and Myrcene. Oval Represent 95% Confidence Limits


AVE3
1.1e6
9e5
7e5
5e5
3e5
1e5
-1e5
4e5
1e6
1.6e6
2.2e6
2.8e6
3.4e6
4e6
AVE4
CONTROL
PRESSURE
HEATED

hexenol, peak 2= α-pinene, peak 3= sabinene, peak 4= myrcene, peak 5= octanal and peak 6= d-limonene.

Figure 28 shows the results of discriminant analysis of the data with the juice treatment determining data grouping. It is clear that discriminant analysis is able to identify differences according to the experimental treatment. The separation of the groups is better than is achieved in principal components analysis since discriminant analysis attempts to weight more heavily the variables that produce group separation. Based on this initial model it is feasible to determine what type of treatment a juice has received, if any.

Table 7 is a listing of the standardized coefficients for canonical variables used in discriminant analysis. The bottom row of Table 7 indicates the cumulative proportion of separation accomplished by each of the roots. Root 1 accounts for approximately 82% of the treatment separation while root 2 explains the remaining separation. When root 2 indicates 100% cumulative separation it means all of the groups are completely separated from each other with 100% classification accuracy. By examining the values for root 1 it can be seen that the highest absolute value of the variable's coefficient belongs to peak 4. This means that peak 4 is the most useful in separating the volatile peak

Figure 28. Discriminant Analysis on Volatiles to Determine Grouping Based on Juice Treatment

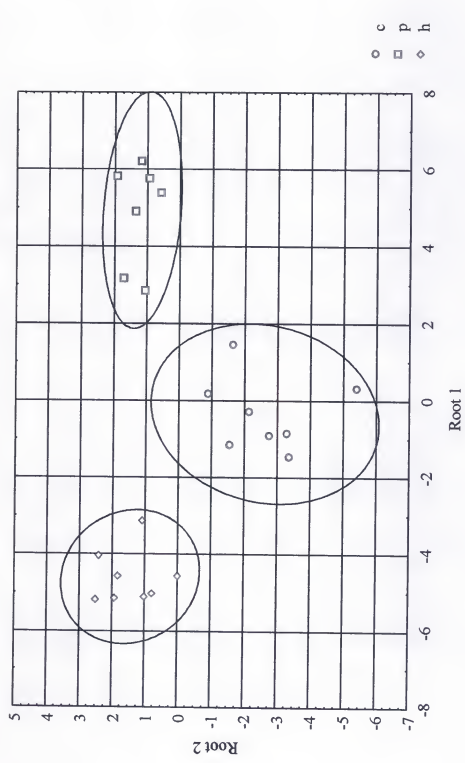

c
p
h
Root 1
Root 2
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
-8
-6
-4
-2
0
2
4
6
8

Table 7. Factor Loadings Used in Discriminant Analysis

Stat. Discriminant Analysis	Standardized Coefficients for Canonical Variables	
Variable	Root 1	Root 2
z-3-hexenol	0.14845	1.44136
α-pinene	1.57206	0.08273
sabinene	0.73635	-1.17316
myrcene	-3.42630	-0.13842
octanal	0.07411	-1.53893
d-limonene	1.55987	0.18202
Eigenvalue	17.37716	3.91985
Cumulative Proportion (%)	0.81594	1.000000

area with regard to treatment. The rest of the coefficients can be ranked in importance based on their relative magnitudes. The results of this ranking is that myrcene is the best indicator of treatment, followed by α-pinene, d-limonene, sabinene, z-3- hexenol and octanal.

The fact that the three treatments are statistically distinguishable from each other indicates that there should be some flavor differences between the three groups. There is a clearly documented flavor change in juice that has been pasteurized (Schreier et al., 1977; Moshonas and Shaw, 1997; Nisperos-Carriedo and Shaw, 1990) but this is not the case with juice treated by high pressure (Parish and Olsson, 1994; Ogawa et al., 1989). Taste panels employing a triangle test on 22 subjects could not discern the difference between pressurized and control Valencia and Navel orange juice. Thus, although the volatile profile is altered in a high pressure processed juice, the flavor is not significantly impacted.

CONCLUSIONS

High pressure was shown to be useful for the inactivation of PE in orange and grapefruit juice. As such, it is a potentially useful tool for extending the shelf life of fresh juice, while preserving its fresh taste and appearance. Results indicate inactivation of the heat labile form of PE, but not the heat stable form. This is possibly due to a higher percentage of hydrophobic amino acid residues in the heat stable form of PE which makes denaturation less favorable and therefore more difficult.

Isoelectric focusing and gel electrophoresis were able to isolate PE from extracted pulp from treated Valencia orange juice. Complete inactivation of PE with pasteurization at 95 °C and alteration of the enzyme with high pressure treatment at 800 MPa and light pasteurization at 75 °C was observed with these methods. A corresponding decrease in pectinesterase activity was noted.

High pressure treatment was also effective in preserving orange juice cloud while maintaining acceptable levels of microbial contamination. In order to achieve a 90 day shelf life of packaged juice at refrigerated

temperatures, pressures of > 700 MPa and processing times of 1 minute or longer are recommended.

Principal component analysis and discriminant analysis were able to differentiate juice treatments by comparing volatile profiles over the course of a 36 week storage study. It was found that α-pinene, sabinene, myrcene and d-limonene were useful indicators of treatments received prior to storage. Discriminant analysis provided more satisfying separation of volatile data based on treatment received prior to storage due to the technique's inherent nature to capitalize on variance between peaks. Flavor of orange juice is not noticeably altered by high pressure, despite the separation of the pressurized sample from the control.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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